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Journal of Pharmaceutical and Biomedical Analysis 36 (2004) 457–464
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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A method for the direct analysis of drug compounds in plasma using a single restricted access material (RAM) column

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Received 16 April 2004; received in revised form 4 July 2004; accepted 12 July 2004
Available online 25 August 2004

Abstract

We describe an automated approach to analyzing whole plasma samples using online extraction without the need for an analytical column. A single restricted access material (RAM) column provided online extraction and pre-concentration of analytes while effectively removing proteins, salts and other biological materials found in the plasma sample matrix. The reduction in the plasma matrix enabled direct elution of the analytes from the extraction column to the mass spectrometer for selective detection. The precision of the method was evaluated using a proprietary therapeutic agent (Compound A) and was less than 5% over the range of 1–500 ng/ml in spiked whole plasma, with an LOQ of 1 ng/ml. A side-by-side comparison of RAM results from a pharmacokinetic study in rats was made with a traditional protein precipitation LC–MS method and a correlation of 0.993 was obtained between both methods. The injection-to-injection cycle time for the RAM method was 8 min. Further automation was demonstrated by addition and mixing of the internal standard to all samples via an injection program of the autosampler.

Keywords: Restricted access material; LC–MS/MS; Direct injection; Online extraction

1. Introduction

Sample preparation is a key consideration for the development of reliable quantitative HPLC methods used to measure therapeutic agents in plasma. However, increasing sample cleanup usually involves a trade-off with increased analysis time, complexity and/or consumables cost. By far the most common approach routinely reported is simple precipitation of plasma proteins using an organic solvent with or without the addition of an acid, followed by centrifugation [1,2]. While simple, this procedure may not provide adequate selectivity since many endogenous plasma components remain in the supernatant and co-precipitation of the analyte in the protein pellet may occur [3]. In addition, the compound of interest is diluted, depending on the amount of organic solvent added, which can impact detection limits. Pre-concentration of the analyte is a benefit afforded by more specific extraction techniques such as solid-phase extraction (SPE), however, the routine use of these methods can be difficult to automate [4].

Alternatively, efforts were made to develop a direct sample injection technique to minimize analysis time and sample manipulations without sacrificing the quality of analysis. A recent report by Mei et al. [5] indicated that sample manipulations can introduce interference from disposable plastic labware. Direct online injection methods may offer the advantage of reducing steps during sample preparation. New column technologies using restricted access materials (RAM) are well suited for this since they combine sample cleanup and pre-concentration in one step, permitting online extraction of whole plasma [6]. In addition to RAM technology, direct online extraction methods have been previously reported using mixed-function columns [7] and online SPE [8]. Typically, RAM columns are used in combination with ana-

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doi:10.1016/j.jpba.2004.07.029

lytical columns, to allow direct injection of plasma without causing irreversible damage to the stationary phase of the analytical column [3,9]. Applications of RAM technology for SPE-LC of bio-fluids have been reported for a wide range of compound classes such as organophosphorus triesters [10], anti-depressants [11], anti-malarials [12] and phthalates [13]. A comprehensive review by Souverain et al., [14] reported over one-hundred applications of direct injection of biological matrices onto RAM supports, however, very few literature examples demonstrated the direct coupling of RAM columns to MS detection.

Our efforts were focused on achieving a reliable method without using an analytical column since MS–MS provides sufficient specificity to achieve this. Furthermore, in addition to minimizing analysis time, eliminating the analytical column also reduces time spent developing the second step in the chromatographic method, which is highly beneficial in early drug discovery.

A RAM column that uses 25 μm silica particles with a C18 phase embedded within the porous center was selected for direct analysis of plasma. Macromolecules greater than 15,000 Da. are prevented from entering the pores of the stationary phase because of size exclusion [1,15]. In addition, the particle surface is also coated with an inert, hydrophilic layer to prevent non-specific adsorption of bio-molecules such as proteins [1,16]. The analyte is retained within the C18 phase during the sample-loading stage permitting removal of the plasma matrix when using highly aqueous mobile phase. Furthermore, a high flow rate during loading effectively removes large bio-molecules and salts. Because of the large particle size (25 μm), back-pressure from the RAM column was well below pressure limits (300 bar) even at flow rates of 5 ml/min. During desorption, a steep gradient was applied since the separation of Compounds A and B was not required for detection and quantitation by MS–MS [17]. In fact, from our experiences with MS–MS quantitation, removal of salts, proteins and other components of the plasma matrix have a much greater impact on data quality and method reliability.

Direct analysis of rat plasma spiked with Compound A and an internal standard (ISTD) Compound B, was evaluated. A pharmacokinetic profile of Compound A dosed in rats was evaluated using both the RAM technique and a protein precipitation method to demonstrate the feasibility of the RAM technique as a routine method.

2. Experimental

2.1. Materials

HPLC-grade methanol and acetonitrile were obtained from EM Science (Darmstadt, Germany). Mass spectroscopy-grade formic acid was purchased from Fluka (Buchs SG, Switzerland). Compounds A and B (Fig. 1) were synthesized in house by the Merck Frosst Medicinal Chemistry group (Montreal, Canada). Deionized water was generated using a Millipore Milli-Q system (Bedford, MA, USA).

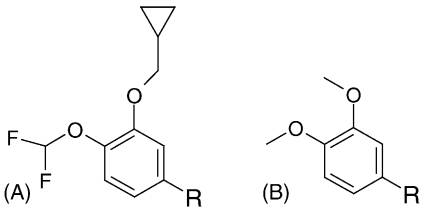

Fig. 1. Partial structures of Compounds A and B (internal standard).

2.2. Equipment

All analyses were performed using an HP1090 HPLC from Hewlett-Packard (Palo Alto, CA, USA) equipped with a Rheodyne (Rohnert Park, CA, USA) switching valve. The online extraction was accomplished using a 25 × 4 mm C18 RAM-ADS (alkyl diol silica) column with a 25-μm particle size, obtained from Merck KGaA (Darmstadt, Germany). An ADS inline filter with a stainless steel frit from Merck KGaA, was installed between the autosampler and the RAM column. Control of the HPLC system was accomplished using Hewlett-Packard Chemstation.

Mass spectrometric measurements were made using a Sciex API III (Thornhill, Canada) mass spectrometer equipped with a heated nebulizer source. Sample scheduling and data capture was performed using RAD™ software and processed using MacQuan from Sciex. Synchronization of the HPLC and MS was accomplished with an Autolink system from MGT systems (Milton Keynes, UK).

2.3. HPLC conditions

Plasma samples (50 μl) were injected onto the RAM column using a mobile phase composition of water/methanol (95:5 v/v) (Solvent A) pumped at a flow rate of 2 ml/min (0–3 min) through the RAM-ADS column and diverted to waste. For analyte desorption, a gradient elution of 0.1% (v/v) formic acid in acetonitrile (Solvent B) at 0.8 ml/min was used, 0–90% B (3–5 min) and sustained at 10% A:90% B (5–7 min) with the eluent flow directed to the mass spectrometer at 4–6.9 min. A short column-recondition stage followed for 60 s (7–8 min) with 100% solvent A at 2 ml/min prior to the start of the next injection. The column temperature was maintained at room temperature (∼25 °C) in the column compartment. Flow to the UV detector was only used during method development to obtain the plasma elution profiles using a UV wavelength of 254 nm (Fig. 2), and to further investigate analyte recovery in the presence of plasma.

2.4. MS conditions

The heated nebulizer source was operated at 450 °C in positive ion mode. The discharge current was optimized to 3 μA.

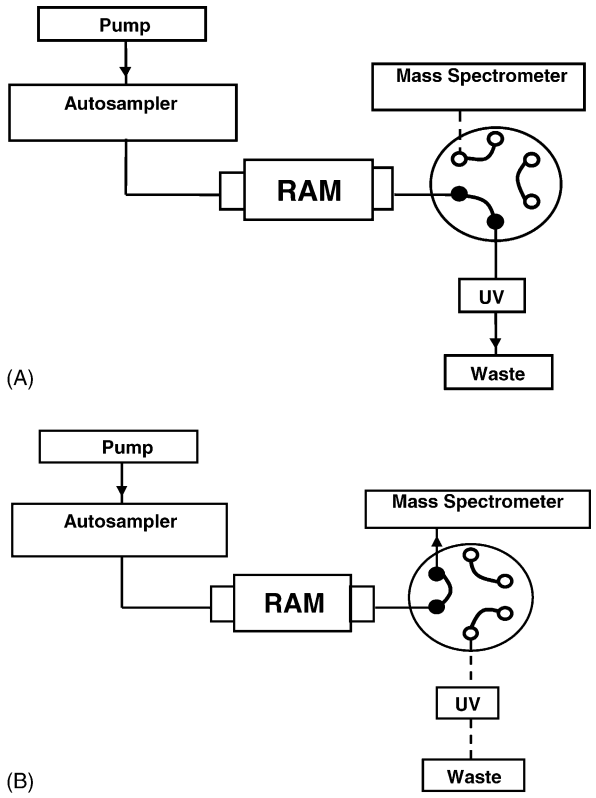

Fig. 2. Schematic of RAM column set-up for direct injection (A) loading (B) desorption/analysis.

High purity nitrogen (99.999%) was used for the curtain gas, nebulizer gas and auxiliary gas, using settings of 0.6 l/min, 65 psi, 2000 cc/min, respectively. Multiple-reaction monitoring (MRM) was used to quantify Compounds A and B. The pause and dwell times were optimized to 20 and 200 ms, respectively. The collision gas (argon) thickness was maintained at an instrumental setting of 220.

2.5. Standard and sample preparations

Standard solutions of Compounds A and B were prepared in water/methanol (50:50 v/v). Each level of the working plasma standards was made by manually spiking 8-parts blank rat plasma with 1-part of the appropriately diluted Compound A standard solution and 1 part internal standard. The final concentrations of the working plasma standards contained approximately 1–500 ng/ml Compound A with ~20 ng/ml internal standard Compound B. All plasma-containing samples were centrifuged at 1000 × *g* for 15 min prior to analysis to remove any suspended materials.

3. Results and discussion

3.1. Method development

Initial method development efforts were focused on maximizing the removal of endogenous plasma components while ensuring complete retention of the analyte. The flow rate during the loading-phase was varied at 1, 2, 3 and 5 ml/min using a mobile phase composition of water/methanol (95:5 v/v). At each flow rate, analyte breakthrough was evaluated after injecting a concentrated standard solution containing 10 μg/ml Compound A in water/methanol (90:10 v/v), while acquiring a UV signal at λ = 254 nm for 1 h. The organic fraction of the extraction solvent was maintained at ≤10% methanol since preparations of plasma-based samples and standards required low levels of organic solvent to prevent protein precipitation during analysis. Achieving sufficient solubility of each drug

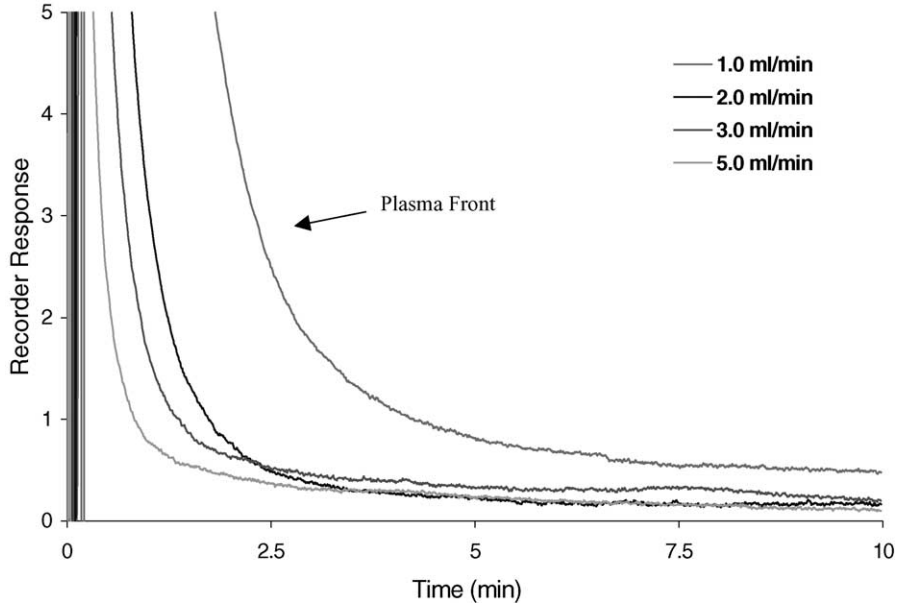

Fig. 3. Injection of 20 μl blank rat plasma using UV detection λ = 254 nm. The flow rate of the mobile phase, water/methanol (95:5 v/v), was varied from 1 to 5 ml/min.

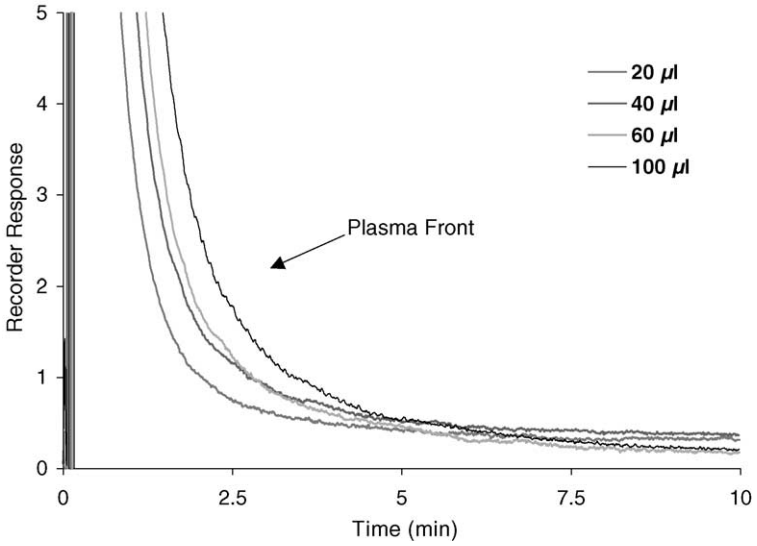

Fig. 4. Injection of 20–100 μl blank plasma using a flow rate of 2 ml/min with UV detection λ = 254 nm. Mobile phase was water/methanol (95:5 v/v).

compound is also a critical consideration when selecting the sample to solvent mixture. Analyte breakthrough was not observed at any flow rate as measured by UV detection at λ = 254 nm (data not shown), indicating successful extraction by the C18 phase within the pores of the RAM particles.

The removal of the plasma matrix was also investigated during the sample-loading phase by varying the loading solvent flow rate (water/methanol 95:5 v/v) at 1, 2, 3 and 5 ml/min after injection of blank rat plasma. Fig. 3 demonstrates the effect of increasing the flow rate during the sample loading stage after a 20 μl injection of blank rat plasma. At flow rates of 2 ml/min and greater, the majority of plasma matrix is removed within 3 min, as shown by a decrease in strongly absorbing plasma front at λ = 254 nm. The dimensions of the RAM column (25 × 4 mm, i.d.) and the large particle size (25 μm), permitted the use of high flow rates which are more effective for sample cleanup. The removal of plasma proteins was further evaluated over a range of injection volumes typically used for bio-analytical samples. Twenty to 100 μl of blank rat plasma were injected using a loading flow rate of 2 ml/min with UV monitoring at λ = 254 nm (Fig. 4). The intensity of the sample front signal increased with the amount of plasma injected, however, the duration required to flush through the column occurred within the same amount of time, as shown by a return to a baseline UV signal. For the routine analysis of plasma samples, a loading flow rate of 2 ml/min was selected and the injection volume maintained at 50 μl. This injection volume was selected as a compromise between the limited plasma volumes available and benefit of sample pre-concentration for improved sensitivity. In order to prevent damage to the RAM column, an inline filter was used to trap particulate materials that may have been present in plasma samples. No changes in performance of the RAM column were observed during the course of the experiments (approximately 300 injections).

Desorption of the drug analytes was accomplished by a gradient elution using 0.1% (v/v) formic acid in acetonitrile. A steep gradient was chosen to facilitate desorption of the analytes from the column, to enable narrow peak shapes and to shorten analysis times. At this stage, the flow rate was reduced to 0.8 ml/min allowing direct coupling to the heated nebulizer source of the mass spectrometer. Fig. 5 shows extracted-ion chromatograms for Compound A, internal standard and blank plasma using this method.

3.2. Precision, accuracy, limit of quantitation and linearity

The precision data is summarized in Table 1, along with the accuracy calculated as a percentage of the expected concentration. Precision was evaluated over a concentration range of 1–500 ng/ml with a total of $n = 3$ injections for each standard level. The RSD of the replicate injections was less than 5% at all levels. The limit of quantitation (LOQ) was established at 1 ng/ml using the lowest spiked standard tested. At this level, a signal-to-noise of ∼17 was achieved. The method was demonstrated to be linear over the entire range tested (1–500 ng/ml Compound A) with a slope of $y = 1.006x$, an intercept of 0.0076 and a determination coefficient (r^2) of 0.99999. Different sources of blank rat plasma were used throughout the validation experiments.

3.3. Recovery

The recovery was determined by comparing the peak areas for Compounds A and B using direct injections of spiked plasma versus injections of neat standards through the RAM column. The results summarized in Table 2 indicate that Compound A shows a decreased recovery in the plasma matrix, while Compound B does not. Changing the ionization mode

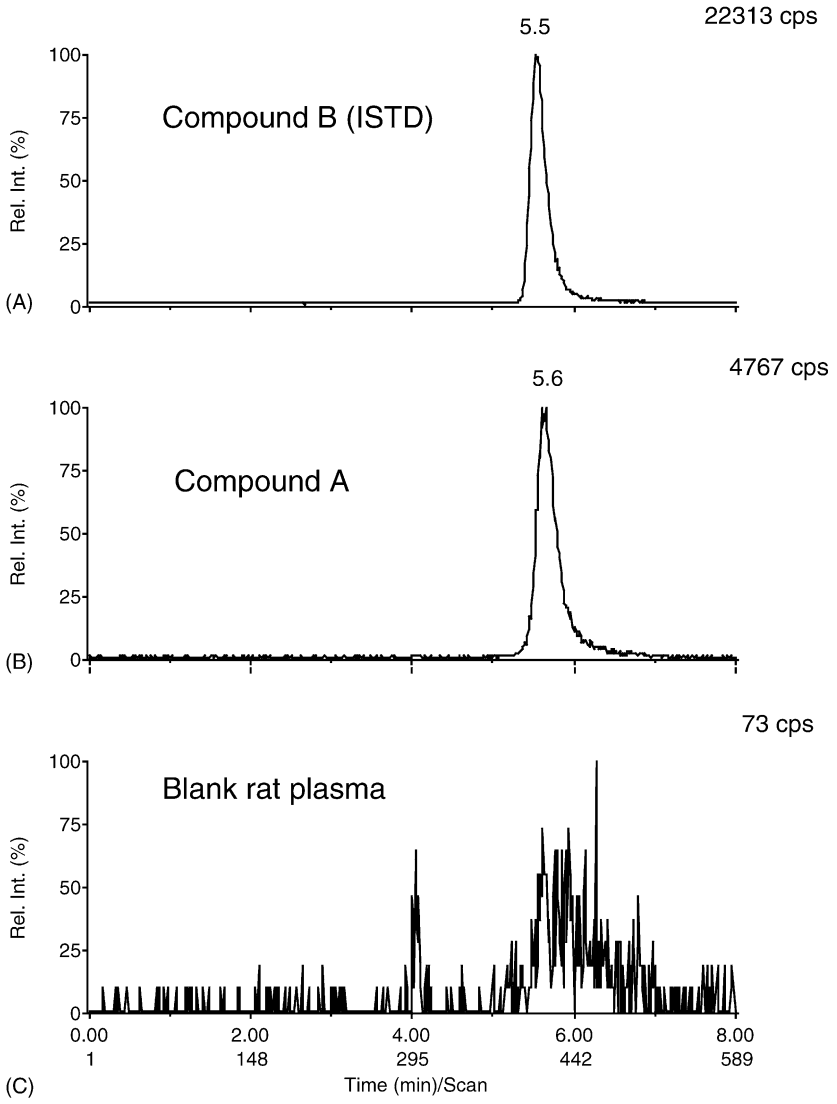

Fig. 5. Extracted-ion chromatogram from a direct injection of 50 μl rat plasma onto a RAM-ADS column (A) Compound B (ISTD) (B) Compound A, 120 ng/ml (C) blank plasma.

Table 1
Performance of RAM column using direct injections of spiked plasma standards

Compound A					
	1.0 ng/ml (n = 3)	4.1 ng/ml (n = 3)	20.4 ng/ml (n = 3)	101.8 ng/ml (n = 2)	509.0 ng/ml (n = 3)
Average	1.2	4.1	21.1	101.5	512.3
Accuracy (%)	115.6	100.7	103.8	99.7	100.6
RSD (%)	3.9	1.9	1.2	–	4.4

Table 2
Recovery of spiked plasma standards extracted using the RAM column as compared to injections of aqueous standards using MS–MS detection

	Compound A						Compound B	
	20 ng/ml		100 ng/ml		500 ng/ml		(ISTD)	
	Plasma	Aqueous	Plasma	Aqueous	Plasma	Aqueous	Plasma	Aqueous
Peak area	27919	68995	181597	401742	846254	2021888	136028	134764
	38440	75674	186721	419081	900078	2047089	134625	133941
Mean	33180	72335	184159	410412	873166	2034489	135327	134353
Recovery (%)	46		45		43		101	

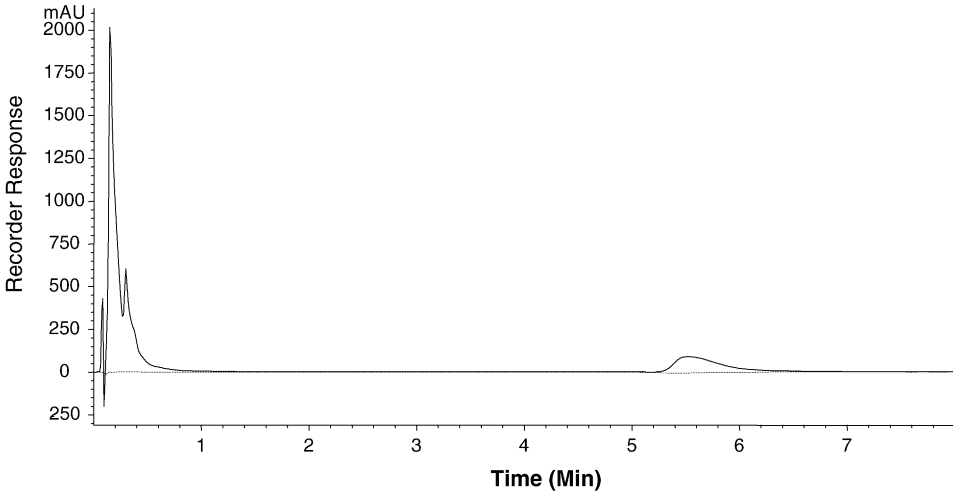

Fig. 6. UV trace at 280 nm using RAM method after a 50 μl injection of blank rat plasma [—] and solvent blank [- - -].

to electrospray (ESI) caused further reductions in recovery (13–21%) of Compound A (data not shown), suggesting that it is likely a matrix effect causing ionization suppression, since ESI is more prone to matrix effects from plasma than APCI [5]. UV detection was employed to further investigate the possible causes for the reduced recovery of Compound A in plasma. The possible reasons include co-eluting impurities causing ion-suppression or non-specific drug–protein binding resulting in the loss of the analyte to waste during the loading stage. Using UV detection at λ = 280 nm, the HPLC chromatogram of a blank plasma injection showed a large amount of unspecified materials eluting in the region of the drug peak (Fig. 6), indicating additional small molecules were extracted from plasma by the C18 phase of the RAM column. By changing to λ = 305 nm, and increasing the gradient elution time, it was possible to quantitate Compound A or B in plasma using UV detection. Recovery was then evaluated by comparing UV peak areas for each compound in water and plasma matrices (see Table 3). MS data were acquired simultaneously and still showed the same recovery trend. The full recovery by UV suggests that the lower plasma recovery observed using MS was because of matrix interference specifically affecting Compound A and not extraction problems associated with the column, arising from non-specific drug–protein binding. Although not investigated in this study, additional commercially available RAM-ADS extraction phases such as RP-4 or RP-8 may have provided better extraction selectivity and less matrix effects for these compounds.

The overall implication of the decreased recovery on method accuracy was further investigated by correlating results to a protein precipitation method that showed full recovery with plasma samples.

3.4. Correlation of pharmacokinetic data

Correlation of the RAM direct injection method to a typical protein precipitation method was made in order to demonstrate accuracy of developed technique. Protein precipitation was achieved using a 1:1 combination of plasma with a 100% acetonitrile containing internal standard, followed by centrifugation at 14,000 rpm for 10 min. Analysis of these samples was performed using a Phenomenex Luna C18(2) 2 × 50 mm, 5 μm particle size analytical column (Torrance, CA, USA) with identical MS/MS detection parameters described for the RAM analysis. The same mobile phase was also used, with a flow rate of 0.8 ml/min and a linear gradient from 10 to 90% B in 8 min (see Fig. 7). The pharmacokinetic (PK) profile of Compound A was measured from plasma samples using both methods after dosing at 3 mg/kg in fasted male rats (n = 4). Fig. 8 shows the PK profiles obtained using both techniques. The curves are essentially identical and the AUC (area under the curve) showed good agreement between

Table 3
Recovery of spiked plasma standards extracted using the RAM column as compared to injections of aqueous standards using UV detection λ = 305 nm and MS–MS

	Compound A (1000 ng/ml)				Compound B (1000 ng/ml)			
	UV λ = 305 nm		MS–MS		UV λ = 305 nm		MS–MS	
	Plasma	Aqueous	Plasma	Aqueous	Plasma	Aqueous	Plasma	Aqueous
Peak area	112.9	105.0	980117	2651648	74.2	78.3	6164118	6352880
	111.1	110.3	959868	2871300	75.2	78.6	6175282	6402188
Mean	112.0	107.7	969993	2761474	74.7	78.5	6169700	6377534
Recovery (%)	104		35		95		97	

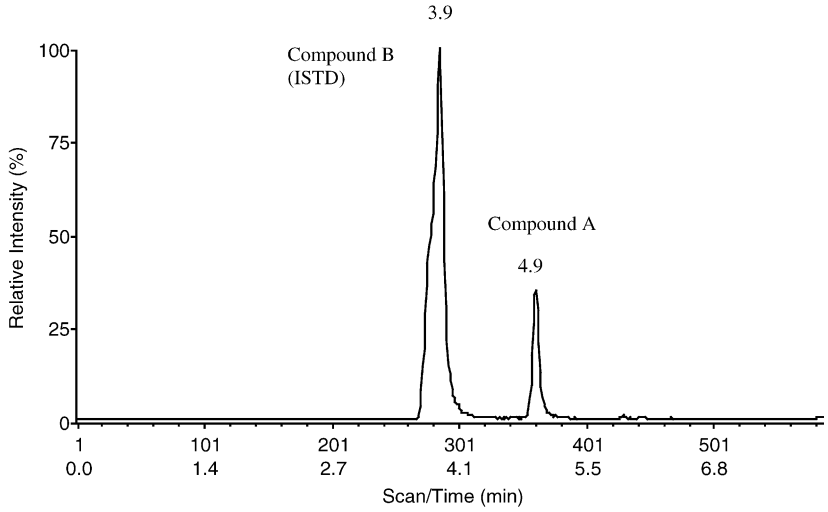

Fig. 7. Total-ion chromatogram from a rat plasma sample after dosing with Compound A, injected onto a reversed-phase (50 mm × 2 mm, i.d.) C18 column after sample pretreatment using protein precipitation. Concentration of Compound A was 120 ng/ml. Flow rate = 0.8 ml/min, mobile phase A water:methanol (95/5 v/v), mobile phase B = 0.1% (v/v) formic acid in acetonitrile, linear gradient of 10–90% B in 8 min.

the direct online extraction and protein precipitation, 2.5 ± 1.0 μg h/ml versus 2.8 ± 0.9 μg h/ml (mean ± S.D.), respectively. A correlation coefficient of 0.993 was found between both sets of results confirming the accuracy of the developed RAM approach, even though a decreased recovery was observed. Any effect of the matrix was therefore compensated by using spiked plasma standards to generate a linear calibration curve.

3.5. Automated addition of internal standard

We also investigated the preparation of plasma samples using a single centrifugation step, since the autosampler was employed to combine the internal standard (IS) to the sample in the correct proportion. The injector program option was setup to draw the IS solution (5 μl), followed by plasma (45 μl) and finally a small volume of air (10 μl) to prevent dripping during mixing cycles. The combination was mixed in the injector loop using 3 mix cycles, prior to injection. This approach was an important use of RAM technology, since it eliminates a tedious step of transferring a correct volume of internal standard to a specified amount of sample plasma. In order to fully benefit from the direct injection of large numbers of plasma samples, tedious preparation steps such as pipetting, mixing and labeling new vials, should be eliminated. The results of the automated addi-

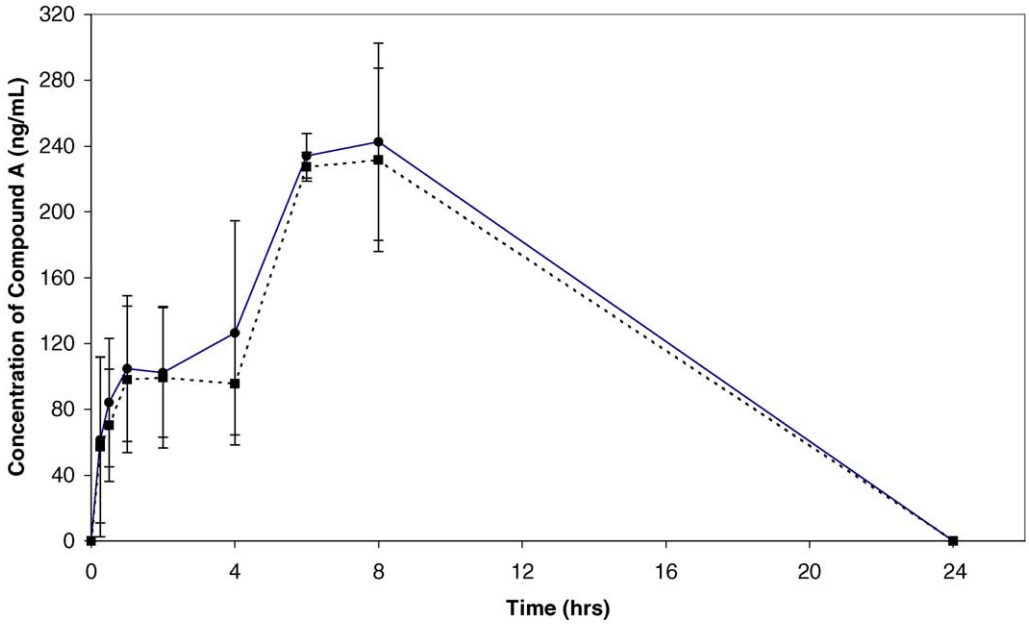

Fig. 8. Comparison of pharmacokinetic profiles of Compound A obtained using protein precipitation [—] and RAM extraction [- - -] methods.

Table 4
Results obtained using automated addition of internal standard to plasma standards

Compound A					
	1.0 ng/ml	4.0 ng/ml	20.0 ng/ml	99.8 ng/ml	399.1 ng/ml
Results (ng/ml)	1.2	3.5	20.6	111.4	389.1
	1.0	3.7	21.1	110.0	405.5
Mean	1.1	3.7	20.8	110.7	397.3
Accuracy (%)	110	93	104	111	99.5

tion are presented in Table 4. The precision and linearity (r^2 = 0.9992) are comparable to the manual additions of the internal standard. However, carryover after numerous injections was observed since many aspirations from the same internal standard vial with the same sampling needle cause contamination in subsequent injections. Further optimization may reduce this effect either using programmed needle washes or drawing from multiple internal standard vials throughout the analysis.

4. Conclusion

RAM technology simplifies method development and is gaining acceptance as an alternative approach for the analysis of bio-analytical samples [18]. It offers an advantage over other extraction techniques by reducing the number of sample manipulations and development time to a few test injections. The technique is easily amenable to analysis of small numbers of analytes but may be more difficult for complex analyses involving quantitation of several metabolites since chromatographic separation is not achieved. If needed, a short analytical column connected downstream of the RAM column may provide some additional improvements in quantitation. Using a pre-column filter is highly recommended to avoid clogging with plasma particulates.

The utilization of the ADS material for other classes of drugs, ensures the potential versatility and suitability of this approach. More fundamentally, the extraction phase located inside the pores of the coating can be designed towards the class of compounds under analysis [19]. Phases with C18 functional groups are more suitable for the extraction of neutral drugs, while the phases with cation exchange properties are more suitable for the extraction of charged compounds like peptides [15] as well as basic drugs [20].

Acknowledgments

The authors wish to thank the Merck Frosst Medicinal Chemistry group for synthesis of the test compounds, Dieter Lubda for helpful discussions and Tony Lee for recovery experiments.

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